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Temporal and spatial expression patterns of Hedgehog receptors in the developing inner and middle ear

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ABSTRACT The mammalian inner ear is a complex organ responsible for balance and hearing. Sonic hedgehog (Shh), a member of the Hedgehog (Hh) family of secreted proteins, has been shown to play important roles in several aspects of inner ear development, including dorsoventral axial specification, cochlear elongation, tonotopic patterning, and hair cell differentiation. Hh proteins initiate a downstream signaling cascade by binding to the Patched 1 (Ptch1) receptor. Recent studies have revealed that other types of co-receptors can also mediate Hh signaling, including growth arrest-specific 1 (Gas1), cell-adhesion molecules-related/down-regulated by oncogenes (Cdon), and biregional Cdon binding protein (Boc). However, little is known about the role of these Hh co-receptors in inner ear development. In this study, we examined the expression patterns of *Gas1, Cdon,* and *Boc,* as well as that of *Ptch1,* in the developing mouse inner ear from otocyst (embryonic day (E) 9.5) until birth and in the developing middle ear at E15.5. *Ptch1,* a readout of Hh signaling, was expressed in a graded pattern in response to Shh signaling throughout development. Expression patterns of *Gas1, Cdon,* and *Boc* differed from that of *Ptch1,* and each Hh co-receptor was expressed in specific cells and domains in the developing inner and middle ear. These unique and differential expression patterns of Hh co-receptors suggest their roles in mediating various time- and space-specific functions of Shh during ear development.

KEY WORDS: *inner ear, hedgehog, Gas1, Cdon, Boc*

The mammalian hearing apparatus consists of the outer, middle, and inner ear. The inner ear is derived from thickened surface ectoderm (also known as the otic placode) on either side of the hindbrain. The otic placode invaginates to form the otocyst, which undergoes a series of morphogenetic changes to produce the three-dimensional structure of the inner ear. The inner ear is composed of six sensory organs, including three cristae (anterior, lateral, and posterior) and two maculae (utricle and saccule) in the vestibule and the organ of Corti in the cochlea. Proper ear development requires multiple signaling molecules, some of which emanate from external sources, such as the neural tube, notochord, and periotic mesenchyme, whereas others are expressed within the otic epithelium itself (Wu and Kelley, 2012).

Sonic hedgehog (Shh) is one such signaling molecule that emanates from surrounding tissues to contribute to multiple steps of inner ear development. During the otocyst stage, Shh is expressed from the floor plate and notochord and specifies the dorsoventral axis of the otocyst, which regionalizes the inner ear into dorsal vestibular and ventral cochlear compartments (Bok *et al.*, 2005, Bok *et al.*, 2007, Brown and Epstein, 2011, Riccomagno *et al.*, 2002). In addition, Shh expressed from spiral ganglion neurons (SGNs) regulates the timing of hair cell differentiation and cochlear elongation (Bok *et al.*, 2013, Tateya *et al.*, 2013). A recent study demonstrated that Shh signaling gradient confers regional identities

Abbreviations used in this paper: Bmp4, bone morphogenetic protein 4; Boc, biregional cdon binding protein; Cdon, cell-adhesion molecule-related/down-regulated by oncogenes; CVG, cochleovestibular ganglia; Gas1, growth arrest-specific 1; Hh, hedgehog; Lfng, LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase; Shh, sonic hedgehog; Ptch1, patched 1; SGN, spiral ganglion neuron.

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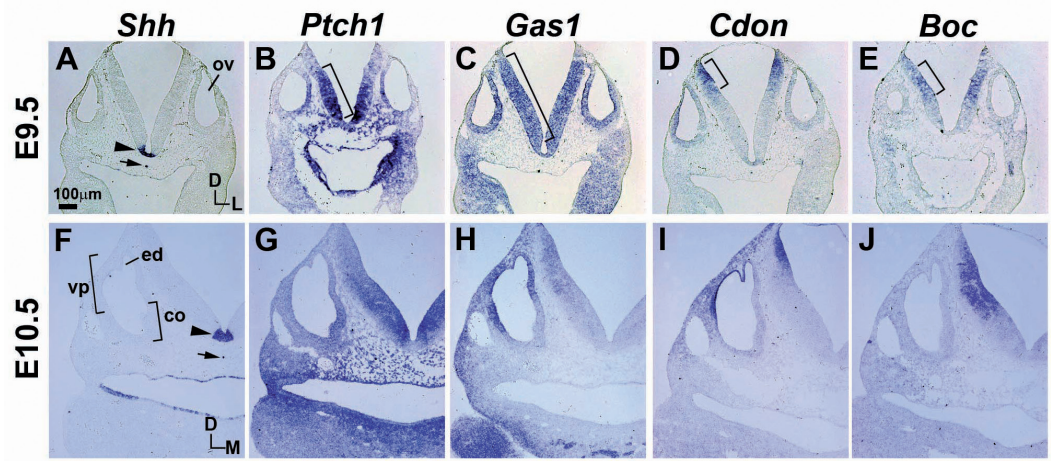
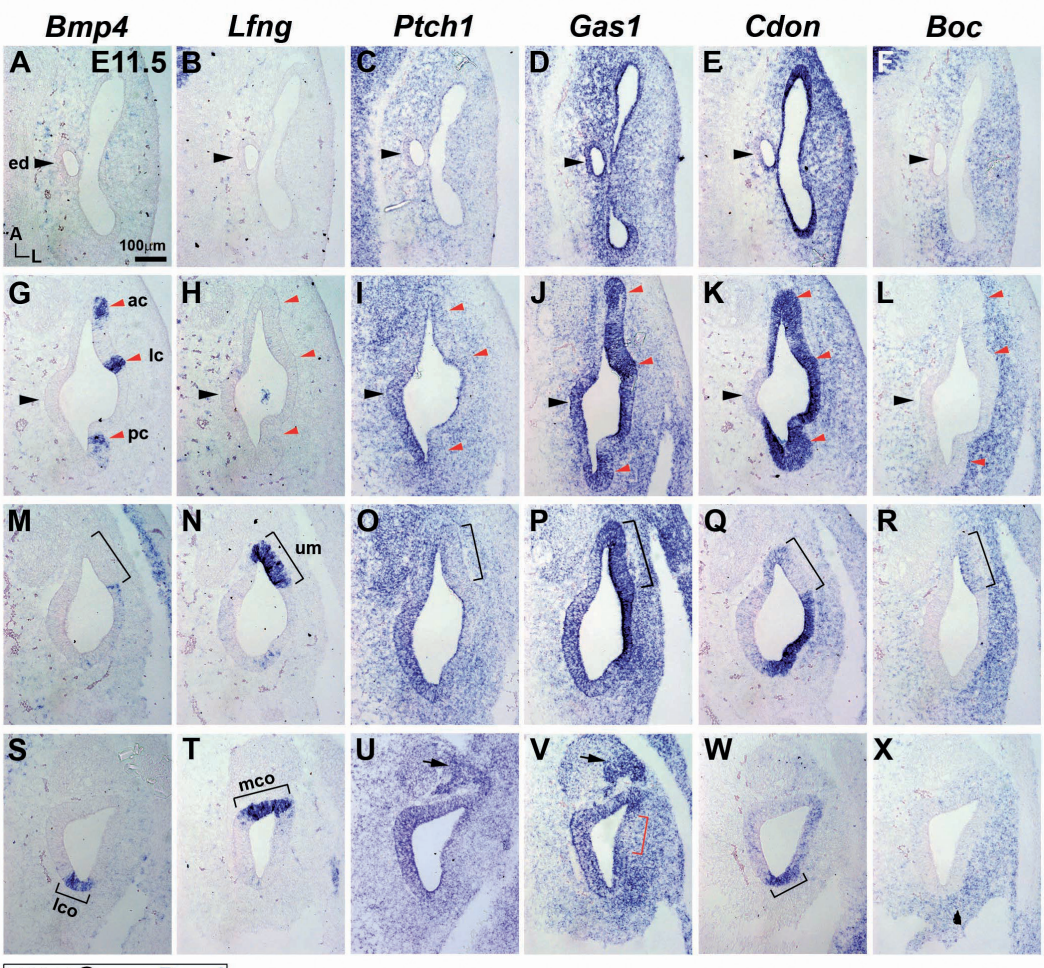


Fig. 1. Expression patterns of hedgehog co-receptors in the otocyst at E9.5 and E10.5. *Expression patterns of* Shh, Ptch1, Gas1, Cdon, *and* Boc *transcripts in the neural tube and otic epithelium at E9.5* **(A–E)** *and E10.5* **(F–J)**. *Square brackets* **(B–E)** *indicate expression domains of Hh co-receptors in the neural tube. Arrowheads and arrows* **(A,F)** *indicate* Shh *expression in the floor plate and notochord, respectively. ov, otic vesicle; co, cochlear duct; ed, endolymphatic duct; vp, vertical pouch; D, dorsal; L, lateral; M, medial.*



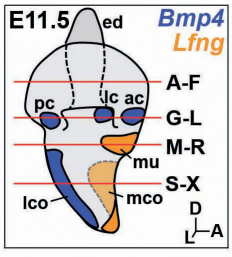


Fig. 2. Expression patterns of hedgehog co-receptors in the inner ear at E11.5. *Expression patterns of* Ptch1, Gas1, Cdon, *and* Boc *in relation to those of* Bmp4 *and* Lfng *in E11.5 otocysts. Horizontal lines in the diagram indicate planes of section, and blue and orange patches indicate expression domains of* Bmp4 *and* Lfng, *respectively. Black arrowheads* **(A–L)** *indicate the developing endolymphatic duct (ed). Red arrowheads* **(G–L)** *indicate anterior (ac), lateral (lc), and posterior (pc) cristae. Brackets* **(M–R)** *indicate the utricular macula (um). Brackets* **(S,W)** *indicate lateral (abneural) cochlea (lco). Bracket* **(T)** *indicates medial (neural) cochlea (mco). Black arrows* **(U,V)** *indicate CVG domain and red bracket* **(V)** *indicates weaker expression domain.* **(X)** *Boc is not expressed in the ventral otic epithelium. A, anterior; L, lateral; D, dorsal.*

to the developing cochlea, which prefigures the tonotopic organization of the mature cochlea (Son *et al.*, 2015).

It is interesting that a single signaling molecule such as Shh exerts such a broad range of functions in a precisely controlled time- and space-specific manner to build a functional inner ear. It is generally accepted that binding of Hh ligands to the receptor Ptch1 activates the key signaling mediator Smo, which initiates a downstream signaling cascade leading to transcriptional regulation by Gli transcription factors (Briscoe and Therond, 2013). Recently, other Hh co-receptors have been shown to play essential roles in mediating Hh signaling. These co-receptors include growth arrest-specific 1 (Gas1), cell-adhesion molecule (CAM)-related/down-regulated by oncogenes (Cdon), and biregional Cdon binding protein (Boc) (Allen *et al.*, 2011). Whereas *Gas1* is a vertebrate-specific gene, *Cdon* and *Boc* are conserved from *Drosophila* to mouse. Cdon and Boc are homologs of *Drosophila* interference hedgehog (Ihog) and brother of Ihog (Boi), respectively, which are functionally and structurally redundant, and are essential for Hh signaling in *Drosophila* (Camp *et al.*, 2010). In mice, Hh co-receptors interact with Ptch1, forming distinct receptor complexes to regulate Hh-mediated function, and simultaneous deletion of *Gas1*, *Cdon*, and *Boc* results

in phenotypes resembling those of *Shh* null mutants (Allen *et al.*, 2011, Izzi *et al.*, 2011, Tenzen *et al.*, 2006). These findings have raised the possibility that Hh co-receptors may mediate various Shh functions required for inner ear development. In this study, we analyzed temporal and spatial expression patterns of *Gas1*, *Cdon*, and *Boc* in the developing inner and middle ear. We observed that each Hh co-receptor was expressed in specific cells or domains, suggesting that distinct combinations of Hh receptor complexes mediate various Shh functions in a time- and space-dependent manner during ear development.

Results and Discussion

Expression patterns of hedgehog co-receptors during early otocyst stages

Expression patterns of *Gas1*, *Cdon,* and *Boc* in the otocyst were examined together with those of *Shh* and *Ptch1* at E9.5 and E10.5 (Fig. 1). *Shh* was expressed in ventral midline structures, such as the floorplate and notochord (Fig. 1 A,F). *Ptch1* was expressed in a graded pattern in the otocyst and neural tube, more strongly on the ventral side and becoming gradually weaker toward the dorsal side (Fig. 1 B,G), representing a Shh signaling gradient emanating from the ventral midline (Bok *et al.*, 2007). Unlike *Ptch1*, *Gas1* was broadly expressed in the otocyst and the neural tube at E9.5, and then was restricted to the dorsal half of the otocyst and neural tube at E10.5 (Fig. 1 C,H). *Cdon* was expressed on the dorsolateral side of the otocyst and in the dorsal tip of the neural tube at E9.5 and E10.5 (Fig. 1 D,I). Expression patterns of *Gas1* and *Cdon* at E10.5 were generally complementary to that of *Ptch1* (Fig. 1 G–I), consistent with the idea that *Gas1* and *Cdon* are negatively regulated by Shh signaling (Allen *et al.*, 2011, Martinelli and Fan, 2007, Tenzen *et al.*, 2006). In contrast, *Boc* expression was not observed in the otocyst at E9.5 and E10.5 (Fig. 1 E,J), and was localized in the middle region between the areas of *Ptch1* and *Cdon* expression in the neural tube (Fig. 1). Gas1, Cdon, and Boc have been shown to play overlapping and essential roles in Shh-mediated ventral patterning of the neural tube by forming distinct receptor complexes with Ptch1 (Allen *et al.*, 2011, Izzi *et al.*, 2011). Comparable patterns of expression of Hh co-receptors in the developing otocyst and neural tube suggest essential roles of Hh co-receptors in mediating Shh functions that are required during early inner ear development, such as dorsoventral axial specification (Bok *et al.*, 2007).

Expression patterns of hedgehog co-receptors in the presumptive sensory regions in the E11.5 otocyst

We next examined expression patterns of Hh co-receptors at E11.5 in relation to the presumptive sensory organs, which can be identified by expression of the markers such as bone morphogenetic protein 4 (*Bmp4*) in cristae and lateral (abneural) cochlea and Lunatic Fringe (*Lfng*) in utricular macula and medial (neural) cochlea (Fig. 2) (Morsli *et al.*, 1998). *Ptch1* was expressed in a graded pattern, more weakly in the dorsal vestibular organs and becoming gradually stronger toward the ventral cochlea (Fig. 2 C,I,O,U). In contrast, *Gas1* was broadly expressed in the otic epithelium and periotic mesenchyme without a clear gradient (Fig. 2 D,J,P,V), suggesting that, unlike *Ptch1*, *Gas1* is expressed independently of Shh signaling at this stage. Interestingly, *Gas1* expression was absent in a small region in the lateral cochlea (Fig. 2V, red bracket), which appears to later develop into the stria vascularis, based on *Gas1* expression at E15.5. *Gas1* was also expressed in the developing cochleovestibular ganglion (CVG) in a pattern similar to that of *Ptch1* (Fig. 2 U,V, arrows). *Cdon* was expressed in a gradient opposite that of *Ptch1* (stronger dorsally and weaker ventrally) in the otic epithelium and periotic mesenchyme (Fig. 2 E,K,Q, W), suggesting negative regulation of *Cdon* by Shh signaling. *Boc* expression was not observed in the otic epithelium, with the exception of an area of weak expression in the utricular macula (Fig. 2 F,L,R,X, bracket). Our results showed that each Hh receptor exhibits unique expression patterns in the E11.5 otocyst. For example, *Gas1* and *Cdon* were expressed in the cristae, whereas *Gas1* and *Boc* were expressed in the utricular macula. In addition, expression domains of *Cdon* and *Boc* demarcated a sharp boundary between the presumptive utricular macula and non-sensory epithelium (Fig. 2 Q,R, brackets). It will be interesting to examine whether distinct combinations of Hh co-receptors contribute to fate determination of specific sensory organs, such as cristae or maculae.

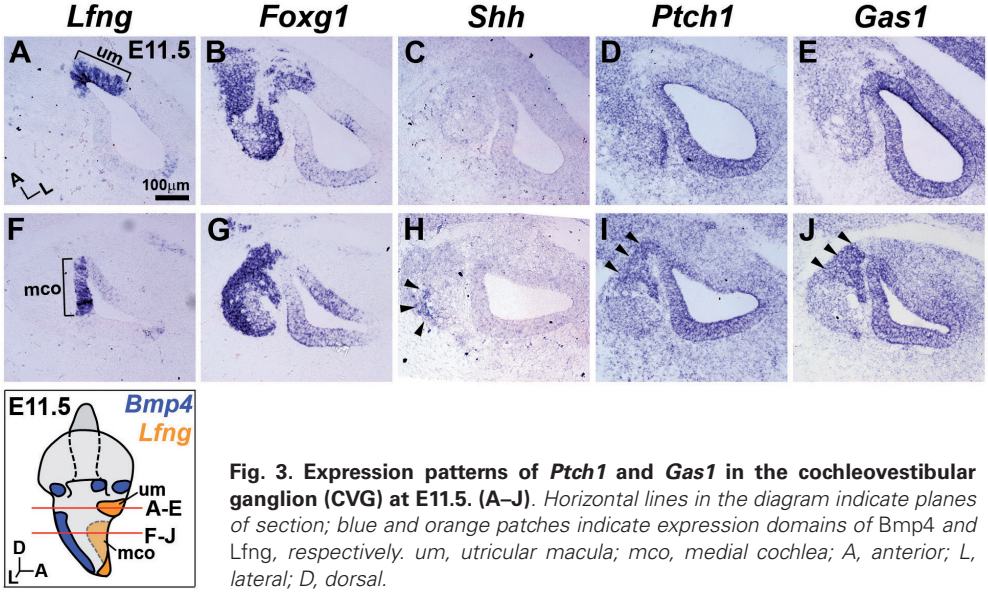


Fig. 3. Expression patterns of *Ptch1* and *Gas1* in the cochleovestibular ganglion (CVG) at E11.5. (A–J). *Horizontal lines in the diagram indicate planes of section; blue and orange patches indicate expression domains of* Bmp4 *and* Lfng, *respectively. um, utricular macula; mco, medial cochlea; A, anterior; L, lateral; D, dorsal.*

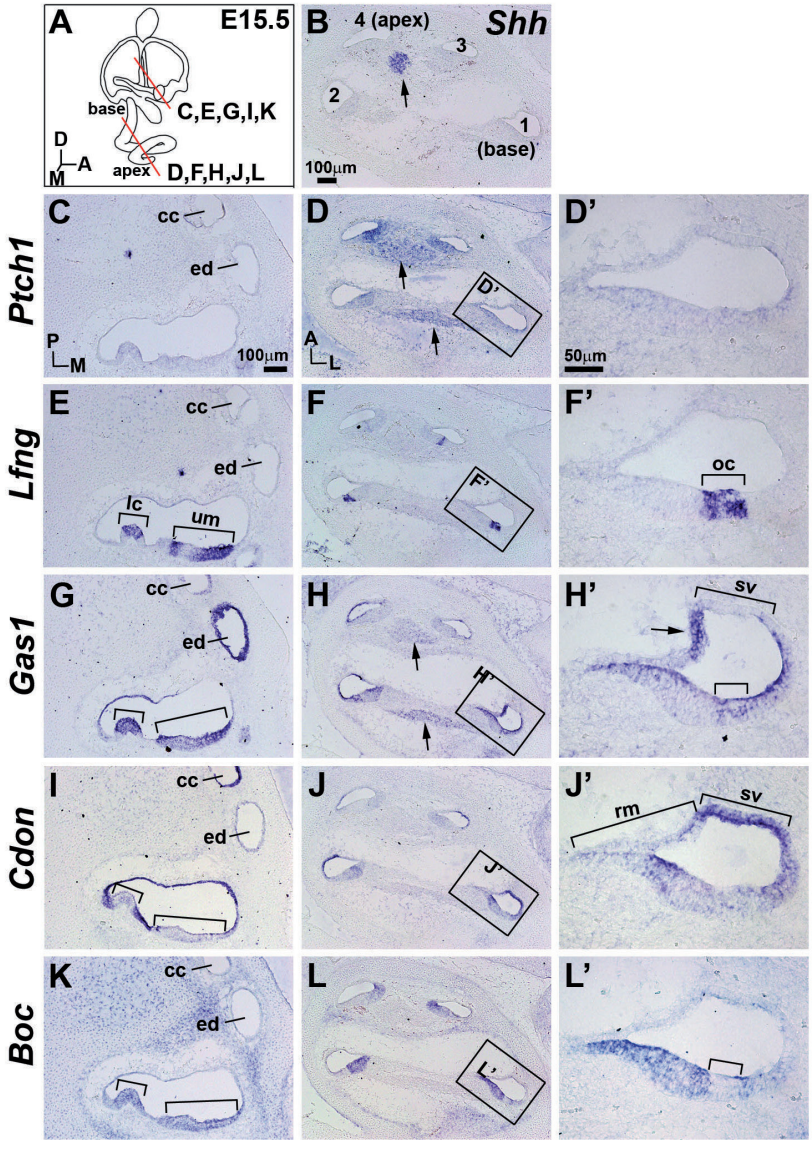
Expression patterns of Ptch1 and Gas1 in the cochleovestibular ganglion

During early inner ear development, neuroblasts delaminate from the otic epithelium to form the CVG, which later connects hair cells to the brain. We analyzed expression of Hh co-receptors in the CVG, which was identified by *Foxg1* expression in the mesenchyme located anteromedial to the otocyst (Fig. 3 B,G). *Shh* expression was observed in the CVG only at the cochlear level and not at the utricular level (Fig. 3 C,H, arrowheads) (Bok *et al.*, 2013). Consistently, *Ptch1* and *Gas1* were upregulated in the CVG located near

the *Shh*-expressing population only at the cochlear level (Fig. 3D, E, I, and J, arrowheads). Neither *Cdon* nor *Boc* was expressed in the CVG (Fig. 2 W,X). Although the roles of ganglionic Shh in hair cell differentiation and cochlear elongation have been well documented (Bok *et al.*, 2013), its role in CVG development is less clear. It will be interesting to examine whether *Ptch1* and *Gas1* mediate Shh functions that are required for CVG development, such as the mitogenic role previously noted in cerebellar granular neuronal progenitors (Izzi *et al.*, 2011).

Expression patterns of Hh co-receptors in the inner ear at E15.5

Expression patterns of Hh co-receptors were examined at E15.5 when the developing inner ear attains its mature morphology (Fig. 4A) and *Lfng* was expressed in all six sensory organs (Fig. 4 E–F') (Morsli *et al.*, 1998). *Shh* was expressed in the SGNs in the apical turn (Fig.4 B, arrow), and *Ptch1* was expressed in the cochlear epithelium and adjacent mesenchyme in a graded pattern that was stronger in the apex and weaker toward the base (Fig. 4D) (Bok *et al.*, 2013). No detectable expression of *Ptch1* was observed in the vestibule (Fig. 4C). *Gas1* was broadly expressed in the inner ear, including the sensory organs, but not in the roof of the utricular macula (Fig. 4G) and the developing stria vascularis (Fig. 4 H–H'), which, interestingly, lacked *Gas1* expression at E11.5 (Fig. 2V). In contrast, *Cdon* was not expressed in the sensory organs, but was expressed in most of the non-sensory regions, with the exception of the endolymphatic duct and Reissner's membrane (Fig. 4J'). *Cdon* expression in the cochlea was graded in a pattern that was stronger at the base and weaker toward the apex, which was opposite that of *Ptch1* (Fig. 4 D,J). *Boc* was weakly expressed in the sensory regions and surrounding mesenchyme in the vestibule (Fig. 4K) and in the Hansen and Claudius cells and greater epithelial ridge (GER) in the cochlea (Fig. 4L'). Our findings showed that the spatial relationships between sensory regions and the expression domains of Hh co-receptors are generally maintained from the otocyst at E11.5 to the inner ear at E15.5, suggesting that Hh co-receptors may be involved in establishment and maintenance of boundaries between sensory and non-sensory regions or between specific inner ear structures, such as Reissner's membrane and stria vascularis.



Expression patterns of Hh co-receptors in hair cells in the neonatal inner ear

We next examined expression patterns of Hh co-receptors in the neonatal inner ear together with that of the hair cell marker atonal bHLH transcription factor 1 (*Atoh1*) (Fig. 5 A–B", arrowheads). No *Ptch1* expression was observed in the inner ear, indicating that little, if any, Shh signaling takes place in the inner ear at this stage (Fig. 5 C–D'). In contrast, *Gas1* was strongly expressed in the vestibular sensory regions, most likely in supporting cells, based on the comparison between *Atoh1* and *Gas1* expression patterns in the cristae and maculae (Fig. 5 A–A" and E–E"). In the cochlea, *Gas1* was broadly expressed in the outer and inner sulcus cells, Reissner's membrane, and supporting cells abutting the hair cells (Fig. 5 F,F'). Unlike E11.5 and E15.5, *Gas1* was expressed in the mesenchyme adjacent to the developing stria vascularis, which appeared to be the differentiating strial basal cell layer (Song *et al.*, 2011). Expression domains of *Cdon* at P0 were continuous with those seen at E15.5, such as in non-sensory

Fig. 4. Expression patterns of hedgehog co-receptors in the inner ear at E15.5. (A) *Red lines in the diagram indicate planes of section.* **(B)** Shh *expression in the spiral ganglion neuron (SGN) of the apical cochlear turn (arrow). Numbers (1–4) indicate the cochlear turns from base to apex. Expression of* Ptch1, Lfng, Gas1, Cdon, *and* Boc *in the vestibule* **(C, E, G, I, K)** *and the cochlear duct* **(D, F, H, J, L)**. *Arrows* **(D, H)** *indicate expression of* Ptch1 *and* Gas1 *in the SGN. The basal cochlear turn is enclosed by black lines and is shown at higher magnification in* **D', F', H', J', L'**. *Small and large brackets* **(E, G, I, K)** *indicate the lateral crista (lc) and utricular macula (um), respectively. Small brackets* **(F', H', J', L')** *indicate the organ of Corti (oc). Larger brackets* **(H' and J')** *represent the stria vascularis (sv) and Reissner's membrane (rm). cc, common crus; ed, endolymphatic duct. A, anterior; D, dorsal; M, medial; P, posterior. Scale bar shown in B applies to panels B–L; Scale bar shown in D' applies to panels D'–L'.*

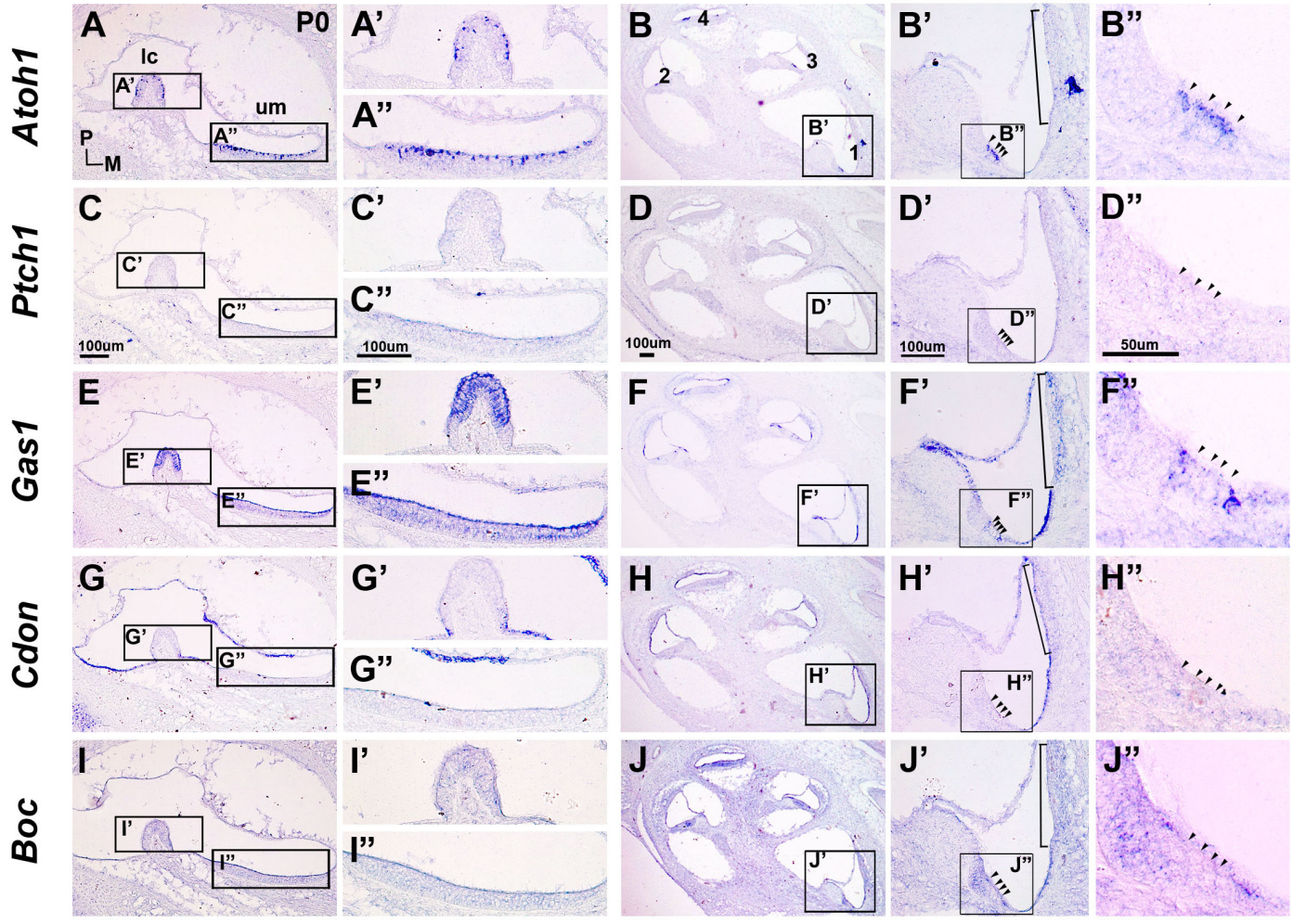


Fig. 5. Expression patterns of hedgehog co-receptors in the inner ear at P0. *Expression patterns of* Atoh1, Ptch1, Gas1, Cdon, *and* Boc *in vestibule* **(A, C, E, G, I)** *and cochlea* **(B, D, F, H, J)**. *The lateral crista (lc) and utricular macula (um) are enclosed by black lines and are shown at higher magnification in* **A′, C′, E′, G′, I′** *and* **A″, C″, E″, G″, and I″**, *respectively. Numbers (1–4) in B indicate the cochlear turns from base to apex. The basal cochlear turn is enclosed by black lines and is shown at higher magnification in* **B′, D′, F′, H′, and J′**; *in these panels the organ of Corti is enclosed in black lines and is shown at higher magnification in* **B″, D″, F″, H″, and J″**. *Arrowheads indicate the hair cells in the organ of Corti. Scale bars apply to all panels in a same vertical column of images.*

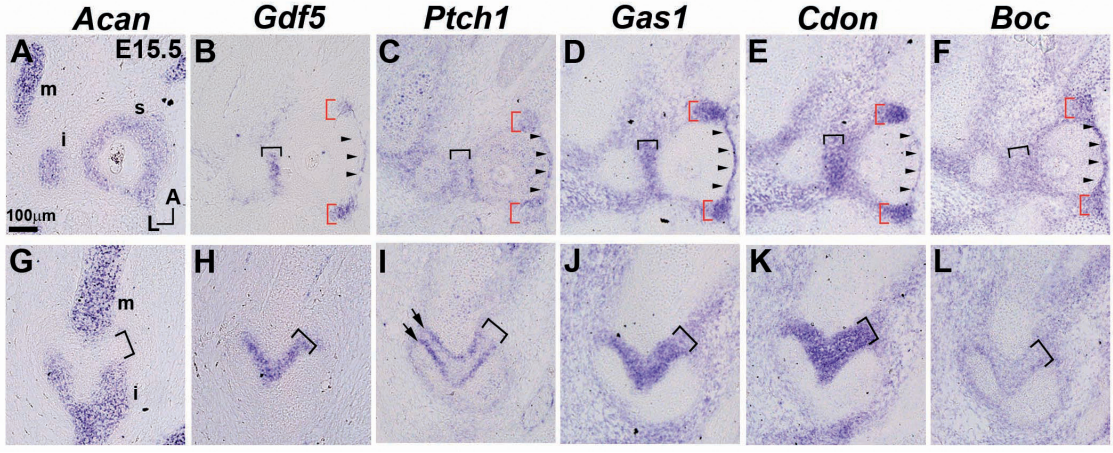


Fig. 6. Expression patterns of hedgehog co-receptors in the middle ear. Acan *is expressed in the middle ear ossicles* **(A,G),** *and* Gdf5 *is expressed in the joints and annular ligaments* **(B,H)**. *Expression of* Ptch1, Gas1, Cdon, *and* Boc *in the middle ear joints (black brackets), annular ligaments (red brackets, B–F), and stapedial footplate (arrowheads, B–F). i, incus; m, malleus; s, stapes; A, anterior; L, lateral.*

regions of the vestibule (Fig. 5 G–G"), outer sulcus cells derived from the lesser epithelial ridge, and strial marginal cells derived from the cochlear epithelium (Fig. 5 H–H'). *Boc* expression was down-regulated in the sensory regions of the vestibule at P0 (Fig. 5 I–I"), but was continuously expressed in the GER (prospective Kolliker's organ), Hensen and Claudius cells, and outer sulcus region (Fig. 5 J–J'). Our results indicated that Hh co-receptor expression domains are generally maintained during inner ear development from E15.5 to P0. However, because expression of *Shh* or *Ptch1* was not detected in the inner ear at P0, it is unclear whether Hh co-receptors contribute to inner ear development by mediating Shh signaling.

Expression patterns of Hh co-receptors in the developing middle ear

The mammalian middle ear consists of a chain of ossicles, including the malleus, incus, and stapes. These ossicles articulate in response to sound vibrations from the outer ear by malleoincudal and incudostapedial joints. The stapes is attached to the cochlea by annular ligaments (Thompson *et al.*, 2012). Since Shh has been shown to be associated with middle ear development (Jeong *et al.*, 2004), we examined the expression of Hh co-receptors in the developing middle ear at E15.5 (Fig. 6). Aggrecan (*Acan*) was expressed in the developing cartilaginous ossicles of the middle ear (Fig. 6 A ,G), whereas growth differentiation factor (*Gdf5*) was expressed in the joints between ossicles and in the annular ligaments between the stapes and cochlea (Fig. 6 B, H, brackets) (Hwang and Wu, 2008). The region of *Ptch1* expression was located between those of *Acan* and *Gdf5* (Fig. 6 C,I). Expression domains of *Gas1* and *Cdon* were similar to that of *Gdf5* in the joints and annular ligaments, but were broader and encompassed both *Gdf5* and *Ptch1* expression domains (Fig. 6 D,E,J,K). In contrast, *Boc* was weakly expressed in a pattern similar to that of *Ptch1* around the joints and annular ligaments (Fig. 6 E,L). Interestingly, none of the Hh co-receptors were expressed in the developing cartilaginous ossicles of the middle ear. It is currently unclear as to what extent Hh signaling contributes to the development of middle ear ossicles and joints. However, because Hh signaling has been shown to play a role in the formation of the temporomandibular joint of the jaw (Purcell *et al.*, 2009), it is possible that Hh co-receptors mediate Hh function to pattern the joints and ligaments in the middle ear.

Summary

In this study, we analyzed spatial and temporal expression patterns of Hh co-receptors, including *Gas1, Cdon*, and *Boc*, in the developing inner and middle ear. Each co-receptor was expressed in specific domains in the inner and middle ear that were generally maintained throughout development, although some specific details changed upon cellular differentiation and morphogenetic change. Our results suggest that Hh co-receptors play essential roles in mediating the functions of Shh, and possibly other Hh ligands, which are required for ear development by forming distinct combinations of receptor complexes in a space- and time-specific manner.

Materials and Methods

Animals

We purchased ICR pregnant mice from KOATECH (South Korea) and harvested the embryos at E9.5, E10.5, E11.5, E15.5, and postnatal day (P)0. All animals were handled in accordance with the Guidelines for the Care and Use of Laboratory Animals of Yonsei University College of Medicine.

In situ hybridization

Antisense RNA probes against *Acan, Gdf5* (Hwang and Wu, 2008), *Atoh1, Ptch1*, *Shh* (Bok *et al.*, 2013), *Bmp4, Lfng* (Morsli *et al.*, 1998), *Boc* [(+2522–+3678), NM_172506.2], *Cdon* [(-127–+949), NM_021339.2], *Foxg1* [(+2428–+3465), NM_001160112.1], and *Gas1* [(+1399–+1878), NM_008086.2] were labeled with digoxigenin (Roche, Mannheim, Germany). *In situ* hybridization was performed as previously described (Morsli *et al.*, 1998). The micrographs of gene expression patterns were taken using OLYMPUS BX40 and Leica DM2500 optical microscopes.

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